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Some Aspects on the Organization of Microfilaments and Microtubules in Relation to Nondisjunction

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One possible mechanism behind nondisjunction is a malfunctioning spindle. A defect or lack of spindle is a criterion for c-mitosis. In chemical mutagenesis research the c-mitotic effect is a well known cytological phenomemon, which can be induced by many different compounds. Pioneer work was performed during the 1940's by Östergren and Levan, and their results and conclusions are briefly discussed. Since colchicine can induce c-mitosis and c-meiosis, by definition, and nondisjunction, a correlation between these phenomena is logical. The general importance of the spindle protein tubulin is considered and some new data from the cell biology literature on spindle formation and function as well as chromosome structure are briefly summarized. This knowledge can be used to correlate cytological and biochemical parameters among which c-mitosis and changes in sulfhydryl group metabolism after chemical treatment are the most obvious ones.

To improve and evaluate test systems for nondisjunction we need information on the biochemical and structural basis for the mechanism of chromosome segregation. This is an attempt to put together some information available in the literature with the aim of recognizing the targets, i.e., molecules or structures of importance in the disjunction process. The action of colchicine and organic mercury is considered to be of principal interest. Some effects of these compounds on the spindle and the chromosomes will be discussed.

Structure and Composition of Spindle Fibers

Microtubules

It has been known since the early 1950s that spindle fibers have an organization which gives rise to birefringence in polarized light. The structure behind this organization and the basic entity in the composition of the spindle fibers most probably is the microtubules (*1*). The molecular unit, which functions as the primary building block for the microtubules is the protein tubulin. This is present in two forms, α- and β-tubulin. In most microtubules, these forms occur in a 1:1 ratio, indicating that the functional unit is a heterodimer (*2*). The assembly and disassembly of microtubules has been studied also *in vitro*, which has given an important insight in the microtubular organization. These studies show that assembly requires GTP and that polyanions like RNA as well as calcium ions may inhibit assembly (Fig. 1).

At the cytological level, the microtubules were previously difficult to visualize in the electron microscope, but altered fixation methods have made this possible in recent years.

A new and important technique to study microtubules in light microscope has been developed. This is done by a fluorescent labeling of antibodies against tubulin. This immunofluorescence technique has been used to study tubulin also in the spindle apparatus.

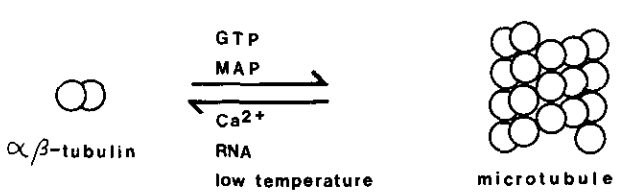

FIGURE 1. Some factors influencing microtubule assembly-disassembly *in vitro*.

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Microfilaments

Beside microtubules, the spindle also contains filamentlike structures, which are formed by actin (*3-5*). It has been suggested on theoretical grounds that myosin also should be present in these filaments and the occurrence of myosin has indeed been reported from studies of the spindle (*6*). Actin and myosin are known as muscle proteins. Their presence in the spindle apparatus indicates a possible role in the chromosome movement and a force generating mechanism similar to the one in muscles has been suggested. There is, however, no clear evidence of the mechanism involved in chromosome movement. Although the important role of microtubules in chromosome segregation can hardly be questioned, there is less agreement about the relevance of actin and myosin in this respect.

Filaments with actin and myosin occur in the cleavage furrow (*3, 6*). Filament structures both in spindle fibers and cleavage furrows are seldom seen in electron micrographs, probably because osmium tetroxide disrupts them (*4*). It has not been shown if microtubules can be chemically bound to microfilaments though a functional interaction has been found in cell growth regulation (*7, 8*).

Microtubule-Associated Proteins (MAP)

When microtubules are isolated and purified for *in vitro* studies some other proteins of interest usually follow along in the purification (*9-11*). These proteins are called microtubule-associated proteins (MAP). They have the ability to facilitate *in vitro* polymerization of microtubules. This does not seem to be a catalytic process, but MAP are incorporated stoichiometrically. One of these proteins has been localized to cytoplasmic structures as well as the spindle with immunofluorescence technique (*10*).

It has been suggested that this type of accessory proteins have something to do with differentiation within the microtubular population of the cell (*11*). There is in fact, a heterogeneity in microtubular composition, which is not unexpected, considering the wide variety of cellular processes dependent on these structures (*12*). The proteins in microtubules and microfilaments, tubulin, actin, and myosin, are subjected to some variation in their amino acid composition (*2, 13*). The designations tubulin, actin, and myosin therefore refer to three groups of proteins. However, the biochemical relationship between the variants within each group is very close.

Cytoskeleton

Microtubules and microfilaments are found in the cytoplasm as a regular network, often referred to as the cytoskeleton. This network is depolymerized before the mitotic spindle is organized (*14*).

The cytoskeleton has been subjected to a series of studies during recent years. An interesting and important outcome of these studies concerns the effect on the cytoskeleton network by cellular transformation. Extensive comparative studies on normal and transformed cells revealed that transformed cells have altered microtubule and microfilament organization (*14, 15*). Although these cells seem to have a normal mitosis, a subtle correlation between the type of transformation and the degree of heteroploidy cannot be ruled out, considering the differences between transformed and normal cells in the microtubule-microfilament organization, some caution may be justified in drawing conclusions from established cell lines, which are transformed in one way or another. We do not know whether these differences between normal and transformed cells may have some relevance considering the regulation of spindle formation and sensitivity to chemicals.

The correlation between transformation and an alteration of the cytoskeleton network indicates that the studies of the cellular units involved in the spindle formation and chromosome segregation may have a wider relevance than could have been foreseen a few years ago.

Microtubules and Microfilaments in the Spindle and the Cleavage Furrow

As mentioned above, cytoplasmic microtubules disassemble before the spindle is formed. Polymerization and arrangement of spindle microtubules then proceeds in a complex way, where the centrioles seem to play a minor or no organizing role at all (*16, 17*). Microtubules from each pole overlap in the middle of the spindle body. Some microtubules may reach the opposite pole. Since microtubules seem to have one end for assembly and show intrinsic polarity, the spindle fibers growing from opposite poles may interact differently than microtubules growing from the same pole (*18*).

Kinetochores have initiating sites for microtubular polymerization. This has been shown in experiments where isolated chromosomes were used (*19*). Recent work also shows that tubulin readily binds to satellite DNA in the presence of MAP. Since kinetochore regions are rich in satellite DNA, the authors suggest that MAP have a role in microtubule assembly onto kinetochores (*20*). Kinetochore fibers are different from nonkinetochore fibers, as indicated by lower sensitivity to changes in temperature (*21, 22*) and treatment with chemicals like colchicine and colcemid (*23*). It is unknown if this heterogeneity among

spindle microtubules depends on intrinsic properties, or depends on something attached to them.

Anaphase separation of chromosomes can be divided into two phases: firstly chromosome movement towards the poles and secondly an elongation of the spindle. Some cells seem to rely on only one type of separation which can be either one. The elongation has been suggested to depend on lateral interaction between nonkinetochore microtubules overlapping in the middle of the spindle body, perhaps connected to each other by some crosslinking molecules (*24*).

Forer found that anaphase movement in spindles from *Nephrotoma suturalis* could be affected by ultraviolet microbeam irradiation without accompanying changes in birefringence and vice versa (*24*). This has led to the conclusion that a force-generating element probably exists which is composed of other molecular species than microtubular proteins, which in turn are considered as the birefringent part of the spindle.

It is most often the movement of the chromosomes towards the poles that has been suggested to rely on contractile proteins. As mentioned above, there is no proof for the significance of contractile proteins in chromosome movement. There are hypotheses which impose no role for contractile proteins: hypotheses based on lateral microtubular interactions combined with changes in the rate of assembly-disassembly reactions (*18, 25*).

Some experiments with the purpose of resolving the mechanism with the use of antibodies have been performed. In one of these experiments, anaphase movement was not inhibited with antibodies to myosin masking the actin-myosin interaction site of myosin (*26*). This probably rules out an actomyosin-dependent anaphase movement. The same antibodies blocked cytokinesis, indicating that contraction in the cleavage furrow is actomyosin-dependent (*26*). It should be pointed out that recent findings indicate that actin may form contractile structures in cytoplasm without myosin and the myosin site of actin is not involved in these structures (*27, 28*). Therefore the role of actin itself in anaphase movement cannot be ruled out.

Antibodies to dynein, a microtubule associated protein found in cilia, seem to block anaphase movement (*29*). In cilia dynein is known to interact with microtubules in a sliding-filament mechanism important to cilium motion (30). This finding may support the hypotheses postulating that anaphase movement has a part which is dependent on interactions between microtubules, yet much work remains to be done before any choice among the existing models can be done.

Chemically Induced C-Mitosis

Östergren and Levan in the 1940s showed on root tip of *Allium cepa* that a lot of different organic chemicals can induce c-mitotic effects (*31*). When comparing these chemicals they found a relationship between water solubility and lowest effective dose (Fig. 2).

These findings indicate the existence of a common mechanism for induction of c-mitotic effects for many different chemicals. This effect may be regarded as unspecific, in the sense that no single target molecule necessarily is involved. Instead the effect can be referred to physicochemical interactions of a more unspecific kind. Some compounds, however, do not fit the regression line. These compounds have a lower threshold value for c-mitotic effects than would be expected from their water solubility. This indicates a more specific action on the cell division process. Among these chemicals with a more specific effect we find organic heavy metal compounds and colchicine.

Colchicine

Colchicine is assumed to have high binding specificity to tubulin (*34*). Therefore it is widely used as a tool for studies on processes where microtubules are involved. Wilson et al. have been able to show the probable mechanism for colchicine action on microtubules (*12, 35*). The effect is substoichiometric, showing that very few colchicine molecules per microtubule are required to inhibit assembly. It has also been shown that colchicine cannot disrupt intact microtubules unless those are in a state of simultaneous assembly and disassembly. Therefore when microtubules show different sensitivity to colchicine *in vivo* this may reflect a difference in rates for the dynamic equilibrium reactions. The mechanism seems to be similar for compounds like vinblastine and podophyllotoxin (*12*).

Compounds Binding to Sulfhydryl Groups

The great importance of balance between sulfhydryl groups and disulfide bonds in spindle formation was suggested by Mazia et al. (*36*). Their hypothesis was soon confirmed, and recent work shows that tubulin has about eight cysteine residues which are important in microtubule formation (*37*).

In addition the assembly state of microtubules is dependent on levels of reduced glutathione. Diamide, probably a specific glutathione oxidizing agent, causes microtubule depolymerization. This has been shown for mitotic spindles as well as for phagocytotic systems (*38*). Although a very complex

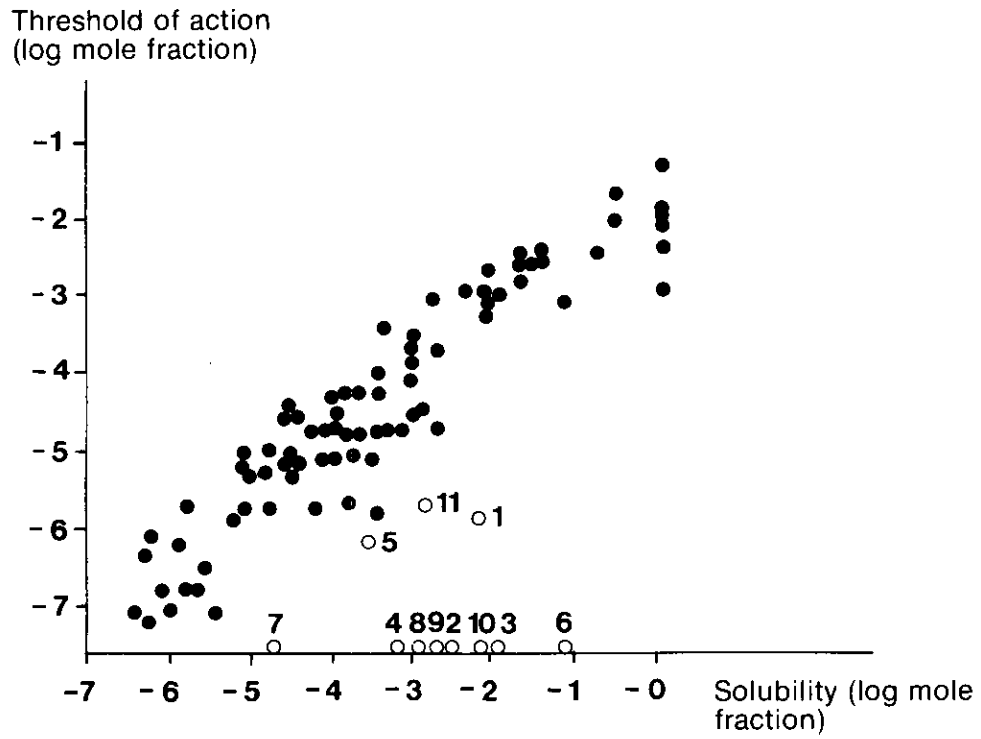

FIGURE 2. Correlation between solubility and lowest concentration for c-mitosis for different organic substances and heavy metals: (1) colchicine; (2) methylmercury dicyandiamide; (3) methylmercury hydroxide; (4) phenylmercury hydroxide; (5) mercury chloride; (6) trimethyltin chloride; (7) tributyltin chloride; (8) diethyllead chloride; (9) triethyllead chloride; (10) trimethyllead chloride; (11) lead nitrate. From Östergren (*32*) and Ramel (*33* and unpublished).

relationship is indicated, the results show that changes in levels of reduced and oxidized glutathione are important biochemical parameters in relation to microtubule disruption.

Some investigated heavy metal compounds are plotted in Figure 2. Among these the organic compounds are active at low dosages. At least the organic mercury compounds show great affinity to sulfhydryl groups and therefore they are expected to interfere with microtubule polymerization (*39*). This can be done in different ways. The most obvious is by binding directly to the sulfhydryl groups on tubulin. Binding to reduced glutathione may give an indirect effect upon microtubule assembly. In addition inactivation of important enzymes cannot be ruled out. When comparing effects of colchicine and organic mercury on Allium root tip mitosis one interesting difference can be found. With colchicine an increase in frequency of c-mitosis cells from a few percent to 100% comes within a narrow concentration interval. Methylmercury hydroxide, however, gives a slower increase towards 100% (*33*). These results probably can be brought back to a higher target specificity for colchicine than for organic mercury.

Chromosome Pairing and Heterochromatin Fusion

Colchicine has been shown to affect meiotic pairing of homologous chromosomes. This was first demonstrated in Allium and then work on Triticum, Lilium and Secale has shown the same phenomenon (*40-42*). These findings implies a role for tubulin or microtubules in meiotic pairing under the assumption colchicine has specific affinity to tubulin.

In Lilium to get an effect on chromosome pairing the treatment had to start before midzygoten. The sensitive period is before nucleolar fusion which starts in zygoten - pachyten in Lilium. The same is valid for Triticum : the colchicine-sensitive period regarding chromosome pairing is just before nucleolar fusion in late G1.

Experiments performed with Secale show that, apart from reduced chromosome pairing, the colchicine treatment also inhibits fusion of heterochromatin usually taking place in premeiotic interphase. A microtubule dependent association of homologous chromosomes before or in meiosis seems to involve heterochromatic regions, e.g., the nucleolar organizing regions. Since these regions are considered

rich in satellite DNA it seems relevant to consider the affinity found for MAP to satellite DNA and the hypothetical role of MAP as mediators of the DNA tubulin contact (*20*).

In connection with this, certain observations on the etiology of Down's syndrome will be noted. Chromosome 21 has a nucleolus organizer region. A significantly higher satellite association was found for chromosome 21 in somatic cells from parents to children with Down's syndrome (*43*).

In addition, it has been shown that persistent nucleoli in somatic cells is a deviation which may lead to disjunction difficulties in anaphase (*44*). These results, taken together, indicate that heterochromatin fusion and satellite associations are important cytological parameters. Changes in pattern for these may be connected with increased probability for nondisjunction.

Chromatin

Nonhistone Proteins

Among the most exciting recent findings are those indicating that tubulin, actin and myosin belong to the nonhistone proteins (NHP) in eukaryotic chromosomes (*45*). The NHP fraction shows variation during cell cycle and is also different in different cell types (*45, 46*). NHP have been suggested to have gene regulative functions (*45*). However, some of them may also fulfill structural tasks. Work recently published by Laemmli's group (*47, 48*) shows that isolated metaphase chromosomes from HeLa cells can be depleted of DNA and histones, but a protein structure still remains. This structure looks like a thin metaphase chromosome and is called scaffold (*49*). One of the proteins in this scaffold shows resemblance to β-tubulin.

Induced Excessive Chromosome Contraction

Colchicine treatment induces excessive chromosome contraction. Assuming that colchicine binds specifically to tubulin, this effect indicates the existence of a tubulin or microtubule dependent part in chromosome folding.

However, according to Östergren, excessive chromosome contraction is found for every compound giving c-mitosis (*32*). This indicates that excessive chromosome contraction is part of the common or unspecific effect induced by any chemical given in a proper dose according to its water solubility. Still colchicine acts in a dose interval suggesting its effect on chromosomes involves a specific target.

The Membrane — A Target for Specific and Nonspecific Actions?

Without going into details, Östergren has pointed out the strong resemblance between the unspecific mechanism behind c-mitosis induction, as defined above, and nonspecific narcosis (*32*). He also stated that the possible structure behind these phenomena would be a lipoprotein complex with important functions, like carrying enzymes or regulating permeability. In addition it has been suggested that the membrane is the site of action for compounds giving narcosis through a nonspecific mechanism (*50*).

Studies on phagocytosis indicate that a complex interaction between cell membrane and cytoplasmic microtubules causes the membrane alterations necessary for phagocytosis. This interaction has been suggested to involve membrane bound tubulin (*51*). Nuclear membranes as well as cell membranes do bind colchicine. Biochemical studies on Lilium meiotic cells show that the main binding capacity is located to the nuclear membrane and the soluble cytoplasmic fraction (*52*). The hypothetical mechanism suggested for membrane alterations in connection to phagocytosis is that membrane bound tubulin increases locally with the aid of cytoplasmic microtubules. This may give rise to a different composition of the cell membrane making it suitable for phagocytosis. In the meiotic cells of Lilium the nuclear membrane bound tubulinlike protein increases in amount as the prophase proceeds. This increase is tied to a change in the composition of the nuclear membrane. In addition the main biochemical effect of colchicine treatment in Lilium was inhibited membrane association of a meiotic DNA-binding protein.

Although it is impossible to suggest any mechanism for colchicine action on the chromosomes, these findings indicate the possible existence of colchicine sensitive alterations also in the nuclear membrane. These alterations then might be of importance to chromosome behavior. Thereby the specific action on mitosis exerted by colchicine would be closely related to the unspecific one exerted by any chemical acting through a mechanism related to unspecific narcosis.

SH Groups in Chromatin Folding

Some recent work in our own laboratory by Irena Klásterská demonstrates that methylmercury hydroxide also has a pronounced effect on chromatin folding in meiotic cells of the grasshopper *Stethophyma grossum* (*53*). The action of this compound may be related to its affinity to sulfhydryl groups. Such groups have been suggested to be of

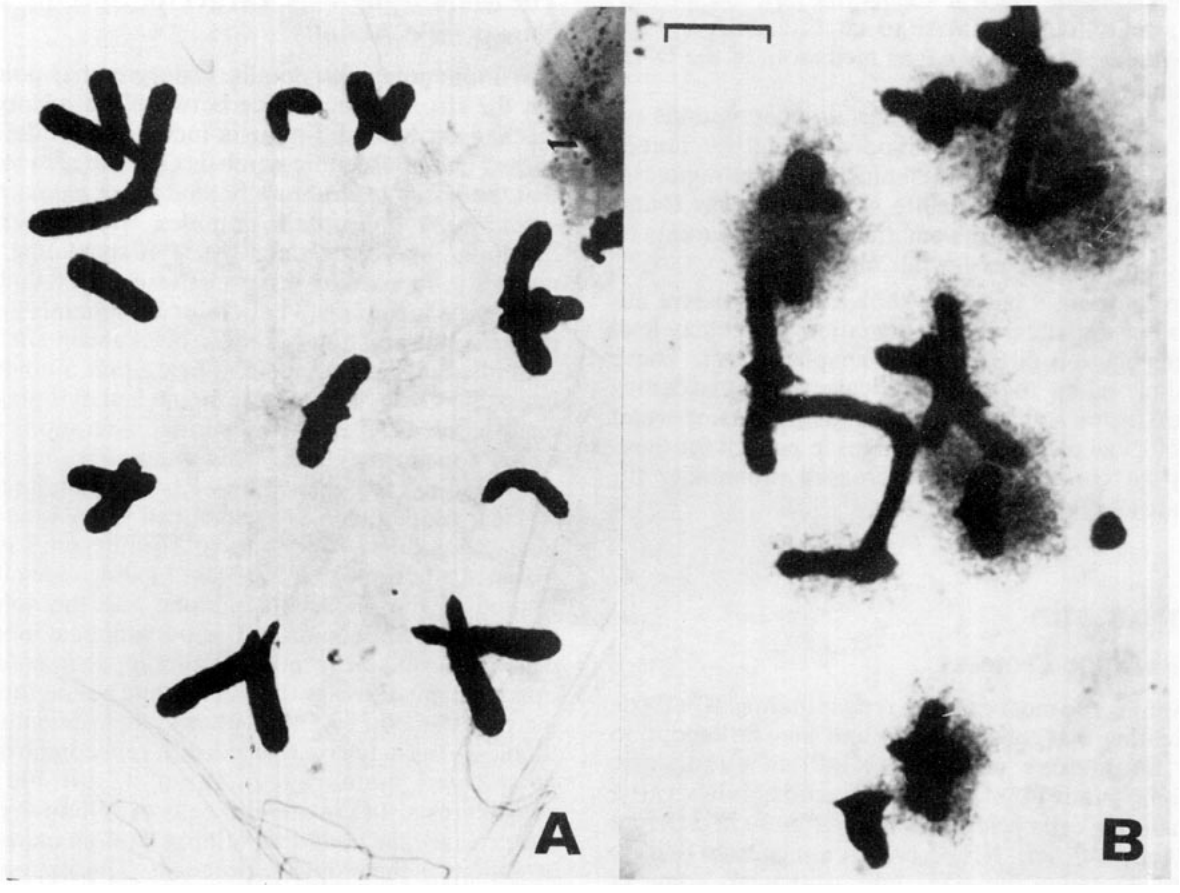

FIGURE 3. *Strephohyma grossum* spermatocyte meiosis stained with modified C-band technique: (A) control; (B) from animal injected with $8 \times 10)^6$ mg CH_3HgOH in Ringer solution. Testis fixed 72 hr after treatment. Bar: 1 μm.

great importance in chromatin folding (*49*). According to this hypothesis the folding process is connected with a change of sulfhydryl groups to disulfide bonds. Histone 3 and nonhistone proteins are suggested to be involved in this folding process. The effect of methylmercury on the folding of the chromatin may therefore be brought back to the same mechanism as operating on the spindle fibers that is, an interference with the oxidation of sulfhydryl groups to disulfide bonds. Cells treated in a stage with already contracted chromosomes can be expected to show less disturbances than those treated during the folding process. That has also been verified by Klăsterská. Metaphase I chromosomes in cells fixed 2 hr after treatment showed no effects on chromatin folding. On the other hand metaphase I cells fixed 6 hr or later after treatment showed gross disturbances in chromosome structure (Fig. 3).

The concentrations used did not induce any excessive chromosome contraction neither in meiotic nor in mitotic cells. However, the folding process of the chromatin evidently was affected and in addition, the disturbance of chromatin folding in mitotic cells was clearly weaker than the one in meotic cells. It is not known if this difference in sensitivity reflects a difference in chromosome organization. Further investigations may show if excessive chromosome contraction and disturbed chromatin folding indeed are interrelated.

Working Hypothesis

Excessive chromosome contraction and spindle disturbances (c-mitosis) can be induced by almost any chemical given in a certain dose related to its water solubility. A common mechanism for most chemicals exists, and this mechanism may act via the nuclear membrane and/or the cell membrane. Some compounds, however, induce c-mitosis at much lower dosages than their water solubility indicates. These chemicals act on specific targets.

The use of colchicine as a specific microtubule inhibitor reveals that microtubules may be important in meiotic pairing, chromosome contraction as well as spindle formation and function.

Changes in pattern of satellite associations and nucleolar behavior may be associated with increased probability for nondisjunction.

Changes in levels of protein sulfhydryl groups and reduced glutathione are important biochemical parameters in disjunction studies.

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